

Distribution of the integral membrane protein NADH–cytochrome b_5 reductase in rat liver cells, studied with a quantitative radioimmunoblotting assay

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The intracellular localization of the post-translationally inserted integral membrane protein, NADH–cytochrome b_5 reductase, was investigated, using a quantitative radioimmunoblotting method to determine its concentration in rat liver subcellular fractions. Subcellular fractions enriched in rough or smooth microsomes, Golgi, lysosomes, plasma membrane and mitochondrial inner or outer membranes were characterized by marker enzyme analysis and electron microscopy. Reductase levels were determined both with the NADH–cytochrome c reductase activity assay, and by radioimmunoblotting, and the results of the two methods were compared. When measured as antigen, the reductase was relatively less concentrated in microsomal subfractions, and more concentrated in fractions containing outer mitochondrial membranes, lysosomes and plasma membrane than when measured as enzyme activity. Rough and smooth microsomes had 4–5-fold lower concentrations, on a phospholipid basis than did mitochondrial outer membranes. Fractions containing Golgi, lysosomes and plasma membrane had ~ 14 -, ~ 16 , and ~ 9 -fold lower concentrations of antigen than did mitochondrial outer membranes, respectively, and much of the antigen in these fractions could be accounted for by cross-contamination. No enzyme activity or antigen was detected in mitochondrial inner membranes. Our results indicate that the enzyme activity data do not precisely reflect the true enzyme localization, and show an extremely uneven distribution of reductase among different cellular membranes.

INTRODUCTION

Integral membrane proteins located in compartments of the exocytic pathway of eucaryotic cells are generally synthesized on membrane-bound polysomes and inserted co-translationally into endoplasmic reticulum (ER) membranes (for reviews see Wickner & Lodish, 1985; Sabatini *et al.*, 1982). There are, however, two well-documented exceptions to this biosynthetic route: rat liver NADH–cytochrome b_5 oxidoreductase (here referred to as reductase) and its electron acceptor cytochrome b_5 are synthesized on free polysomes and inserted post-translationally into membranes (Borgese & Gaetani, 1980, 1983; Rachubinski *et al.*, 1980; Okada *et al.*, 1982). Both of these integral membrane proteins are characterized by (i) large hydrophilic, cytoplasmically located, domains, and short hydrophobic stretches anchoring them to the membrane (reviewed by De Pierre & Dallner, 1975) and (ii) a relatively wide subcellular distribution (Borgese & Meldolesi, 1980; Sottocasa *et al.*, 1967). There is, however, considerable disagreement on the exact subcellular localization of these two proteins. While one group believes that they are localized exclusively to mitochondrial outer and ER membranes (Wibo *et al.*, 1981), we have previously demonstrated their presence within a Golgi-enriched fraction, on elements distinguishable from contaminating microsomes (MR) and mitochondrial outer membranes (OMM) on the basis of density perturbation experiments (Borgese &

Meldolesi, 1980). Others have reported high levels of cytochrome b_5 as well as of the reductase in liver plasma membrane (PM) fractions (Jarasch *et al.*, 1979).

A better knowledge of the subcellular distribution of these post-translationally inserted proteins would help to understand the mechanism of their integration into membranes. In other words, are these proteins specifically inserted only into a set of acceptor membranes, or can they partition non-specifically into any lipid bilayer? Indeed the latter possibility has often been suggested (e.g. Lodish *et al.*, 1981).

As far as the reductase is concerned, much of the uncertainty regarding its localization can be attributed to the exclusive use of enzyme assays to study its subcellular distribution, because: (1) the enzyme might be present in some locations in an inactive form or become inactivated during cell fractionation (Borgese & Meldolesi, 1980); (2) unrelated enzyme proteins could contribute to the measured activity; (3) the enzyme assay generally used, NADH–cytochrome c reductase, measures the transfer of electrons from the reductase to cytochrome c via an intermediate electron carrier (cytochrome b_5). Since the concentration of endogenous intermediate acceptor is not saturating (Strittmatter *et al.*, 1972), differences in its concentration in different compartments could cause differences in activity at equal reductase concentrations.

Because of these problems, we have reinvestigated the subcellular distribution of rat liver cytochrome b_5 reductase, using an immunological method to measure

Abbreviations used: ER, endoplasmic reticulum; G_{1+2} , combined light and intermediate Golgi fraction; IMM, inner mitochondrial membrane; LYS, lysosomes; MAO, monoamine oxidase; MITO, mitochondria; MR, microsomes; OMM, outer mitochondrial membrane; PM, plasma membrane; r.s.a., relative specific activity; RMR, rough microsomes; SMR, smooth microsomes; TH, total homogenate; ws-red, water-soluble fragment of NADH–cytochrome b_5 reductase.

its concentration and comparing the distribution of the antigen with that of the enzyme activity. The results show that indeed the two distributions are markedly different and that the reductase is distributed very unevenly among different compartments, raising questions concerning the mechanisms by which the cell regulates the concentration of this enzyme in different membranes.

MATERIALS AND METHODS

Materials

In addition to reagents used in previous studies (Borgese & Gaetani, 1983; Borgese & Meldolesi, 1980; Borgese *et al.*, 1982), the following chemicals were purchased from the sources indicated below: metrizamide, analytical grade, Nyegaard and Co. A/S, Oslo, Norway; tryptamine HCl, BDH; [6-³H]tryptamine HCl, uridine diphospho-D-[U-¹⁴C]galactose and Na¹²⁵I, Amersham International. Other chemicals were from Sigma Chemical Co.

Cell fractionation

Centrifugations were carried out in Spinco-Beckman ultracentrifuges and in a Sorvall RC-5B refrigerated superspeed centrifuge.

Male Sprague-Dawley rats, weighing 150–220 g and starved overnight, were killed by decapitation. The livers were removed and immersed in ice-cold homogenization buffer. All subsequent operations were carried out at 4 °C. In most experiments, blood was removed from the livers by retrograde perfusion with homogenization buffer. In the PM experiment perfusion was with 0.15 M-NaCl. No perfusion was carried out in the lysosome (LYS) experiments.

In all the cell fractionations, except for the PM experiment, an MR fraction was prepared by sedimentation from the postmitochondrial supernatant at 170 000 *g*_{max} for 1 h.

In all experiments, volumes of samples at each step were registered, and aliquots were kept, in order to run balance sheets and calculate recoveries of all constituents measured. Recoveries of 80% or more were considered acceptable.

Rough and smooth microsomes and Golgi were prepared as previously described (Borgese & Meldolesi, 1980) using buffered sucrose solutions, but an extra layer of 1.6 M-sucrose was introduced in the discontinuous sucrose gradient. Rough microsomes (RMR) were collected in the 1.6 M-sucrose layer and the interface between the 1.6 M and 2.0 M-sucrose layers, smooth microsomes (SMR) at the interface between the load zone and the 1.1 M-sucrose layer, and G₁₊₂ [corresponding to the combined light and intermediate Golgi fraction of Ehrenreich *et al.* (1973)] at the 0.86/0.25 M-sucrose interface.

Lysosomes and plasma membranes were prepared as described by Wattiaux *et al.* (1978) and Hubbard *et al.* (1983), respectively.

A mitochondrial fraction was prepared by differential centrifugation, essentially as described by Parsons *et al.* (1966), using 9 vol. of 0.25 M-sucrose/0.1 mM-EDTA/1 mM-Tris/HCl, pH 7.4, to homogenize the livers in a Potter-Elvehjem homogenizer (three strokes at 120 rev./min), and 0.4 M-sucrose, containing the same ions, to wash the crude mitochondrial pellets.

To prepare OMM, the mitochondrial fraction was subjected to three cycles of hypo-osmotic lysis and centrifugation as described by Mihara *et al.* (1982), using 10 mM-Tris/HCl, pH 7.5 (5 ml/g of liver) as hypo-osmotic buffer. The material extracted by the hypo-osmotic treatment was recovered by centrifugation (16 500 rev./min for 40 min in the SA 600 rotor), resuspended by hand homogenization in 0.25 M-sucrose/0.1 mM-EDTA/1 mM-Tris/HCl, pH 7.4 (0.2 ml/g of liver), diluted with 2.1 vol. of 2.0 M-sucrose (containing the same ions as the homogenization buffer), and loaded under a discontinuous sucrose gradient in centrifuge tubes of the Beckman SW 27 rotor consisting of the following layers (all containing 0.1 mM-EDTA/1 mM-Tris/HCl, pH 7.4): 10 ml of 1.12 M-sucrose, 10 ml of 0.805 M-sucrose, and enough 0.25 M-sucrose to fill the tube. After centrifugation at 26 000 rev./min for 2 h, the OMM band was collected at the interface between the 1.12 M- and 0.805 M-sucrose layers.

To prepare an IMM+matrix fraction, the mitochondrial fraction was subjected to two cycles of the digitonin treatment originally described by Levy *et al.* (1966). The ratio (w/w) of digitonin to mitochondrial protein was 0.13.

Biochemical assays

Enzyme assays were carried out either on fresh material or on fractions stored in suspension at –80 °C for up to 1 month. The activity of the enzymes we assayed was not affected by storage under these conditions nor by freeze-thawing one time. The proportionality of the reaction velocity to enzyme concentration was routinely checked in each assay with all samples.

The following enzyme activities were assayed by the indicated published procedures: rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.2.2) and NADPH-cytochrome *c* reductase (EC 1.6.2.4), Sottocasa *et al.* (1967); esterase (EC 3.1.1.2), Beaufay *et al.* (1974); monoamine oxidase (EC 1.4.3.4), Wurtman & Axelrod (1963) as modified by Shore & Tata (1977); β -galactosidase (EC 3.2.1.23), Vaes (1966), using 50 mM-sodium citrate buffer, pH 3.6, containing 0.2% Triton X-100, as incubation medium, and the substrate (*o*-nitrophenyl galactoside) at a concentration of 10 mM; arylsulphatase (EC 3.1.6.1), Roy (1960), using 0.125 M-sodium acetate buffer, pH 6.0, containing 0.1% Triton X-100, as incubation medium, and the substrate (*p*-nitrocatechol sulphate) at a concentration of 11 mM.

Galactosyl transferase (EC 2.4.1.38) was assayed as previously described (Borgese & Meldolesi, 1980), but fetuin, desialylated and degalactosylated as described by Kim *et al.* (1971), was used as acceptor and the reaction was stopped by addition of 3.0 ml of 1% phosphotungstic acid in 0.5 M-HCl. Alkaline phosphodiesterase I (EC 3.1.4.1) was assayed in 125 mM-Tris/HCl, pH 8.9, containing 5 mM substrate (*p*-nitrophenylthymidine 5'-phosphate). The reaction was followed by monitoring the development of the yellow colour at 400 nm. Cytochrome oxidase (EC 1.9.3.1), was assayed by following the reoxidation of cytochrome *c* previously reduced with NaBH₄ (Sottocasa *et al.*, 1967). Fractions were preincubated (15 min or longer) with digitonin (0.2 mg/mg of protein) before the assay.

Phospholipid phosphorus was determined on lipid extracts (Folch *et al.*, 1957) as described by Rouser *et al.*

(1970). RNA was determined by the modified Schmidt-Tannhauser procedure described by Munro & Fleck (1966). Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Fractions from metrizamide gradients were first precipitated with trichloroacetic acid, using 0.75 mg of deoxycholate as coprecipitant to ensure complete recoveries (Bensadoun & Weinstein, 1976).

Purification of proteins and production of antibodies

The preparation of the water-soluble fragment of the reductase (ws-red) and the affinity purification of antireductase antibodies raised in rabbits have been described in previous publications (Borgese *et al.*, 1982; Meldolesi *et al.*, 1980). Purified ws-red was stored in aqueous solution (~5 mg/ml) at -20 °C in small portions, and used as standard in the radioimmunoblotting assay (see below). It was found that the Lowry assay underestimated the reductase concentration by 35% and values were corrected accordingly. Protein concentration was measured on each standard immediately before use.

A partially purified preparation of rat liver microsomal cytochrome *b₅* was obtained by following the procedure of Omura *et al.* (1967) through the gel filtration step. The resulting preparation was devoid of NADH-cytochrome *c* reductase activity. The concentration of cytochrome *b₅* was determined by the difference spectrum between the oxidized and the reduced form (Garfinkel, 1958).

SDS/polyacrylamide-gel electrophoresis and quantitative radioimmunoblotting

SDS gel electrophoresis of reduced alkylated samples was carried out as previously described (Borgese *et al.*, 1982) on 10% polyacrylamide slab gels 1.5 mm thick. Samples containing purified standard ws-red were supplemented with bovine serum albumin to a final concentration of 0.1 mg/ml. To prevent merging of lanes a comb was used in which sample slots 5.5 mm wide were alternated with smaller slots 2.5 mm wide, which contained only loading buffer.

Electroblotting was carried out in a home-made apparatus, with platinum wires, running up and down the side of the box twice, as electrodes. The blotting box was built into a larger perspex box, which was connected to the tap for water cooling. Blotting was carried out in a methanol-containing buffer (Towbin *et al.*, 1979) for 1.5 h. A voltage gradient of 20 V/cm was applied, resulting in an initial current of 0.5 A, which increased to 0.8 A by the end of the run. The gel heated considerably during this period (60 °C), notwithstanding the water-cooling device. Radioimmunostaining of the blots with antireductase antibodies and iodinated protein A was carried out as detailed previously (Borgese *et al.*, 1982), but incubation with antibody (3 µg/ml) was for 2 h at room temperature. After immunoblotting, the positions of the reductase bands were determined by superimposing an autoradiogram of the blot on the nitrocellulose filter; the bands were then excised and quantified by scintillation counting. A background of ~1000 c.p.m., corresponding to the radioactivity contained in an area of equal size excised from a region of the blot devoid of bands, was subtracted. In most experiments, only the region of the gel containing the reductase band was electroblotted. We routinely blotted 21 samples onto an 11 cm × 11 cm nitrocellulose filter.

Electron microscopy

With the exception of the *G₁₊₂* fraction, subcellular fractions were fixed in suspension with glutaraldehyde (at a final concentration of 2%) in 0.12 M-cacodylate buffer, pH 7.2, and then processed for electron microscopy by routine procedures (Borgese & Meldolesi, 1980). The *G₁₊₂* fraction was fixed in suspension with 2% OsO₄ and processed as previously described (Borgese & Meldolesi 1980).

RESULTS

Characterization of liver subcellular fractions

The subcellular fractions prepared for this study were characterized both morphologically and biochemically. The ultrastructural analysis revealed the expected components for the different fractions (Fig. 1). The results of the biochemical analyses are shown in Tables 1 and 2. The data of Table 1 refer to the chemical composition of the fractions, while the results of marker analyses are given in Table 2.

Because of the reduced levels of plasma proteins in homogenates prepared from the perfused liver, the specific activities of hepatocyte enzymes (on a protein basis) were higher in these homogenates than in their nonperfused counterparts, and the relative specific activities in subcellular fractions were correspondingly lower. When this difference is taken into account, our subcellular fractions showed enrichments in their putative marker enzymes which are in general agreement with the reported values in the literature. An exceptionally high enrichment in MAO was observed in the OMM fraction (~16-fold over the starting MITO fraction), indicating that a high degree of purification of OMM was obtained.

Activity of cytochrome *b₅* reductase, measured as NADH-cytochrome *c* reductase, in subcellular fractions

The first column of Table 3 shows the r.s.a. data for NADH-cytochrome *c* reductase, the enzyme activity generally considered as an indicator of reductase concentration. In agreement with previous studies (Borgese & Meldolesi, 1980; Jarasch *et al.*, 1979; Sottocasa *et al.*, 1967; Wibo *et al.*, 1981), this enzyme activity showed a distribution different from that of classical ER markers, in that it was relatively less concentrated in MR, and more concentrated in the other subcellular fractions, especially the mitochondrial fraction. Within the mitochondrial fraction, its distribution closely paralleled that of MAO, showing an ~18-fold enrichment in the OMM fraction over the starting mitochondrial fraction and confirming its outer membrane localization. The r.s.a. (on a protein basis) of NADH-cytochrome *c* reductase in the purified OMM fraction was thus much higher (~7-fold) than that of crude MR.

Quantitative radioimmunoblotting assay for the determination of reductase concentrations in liver subcellular fractions

In preliminary experiments, we determined that, under our blotting conditions, all Coomassie Blue-stained polypeptides with apparent *M_r* values below 40000, including the ws-red (apparent *M_r* 32000), were eluted

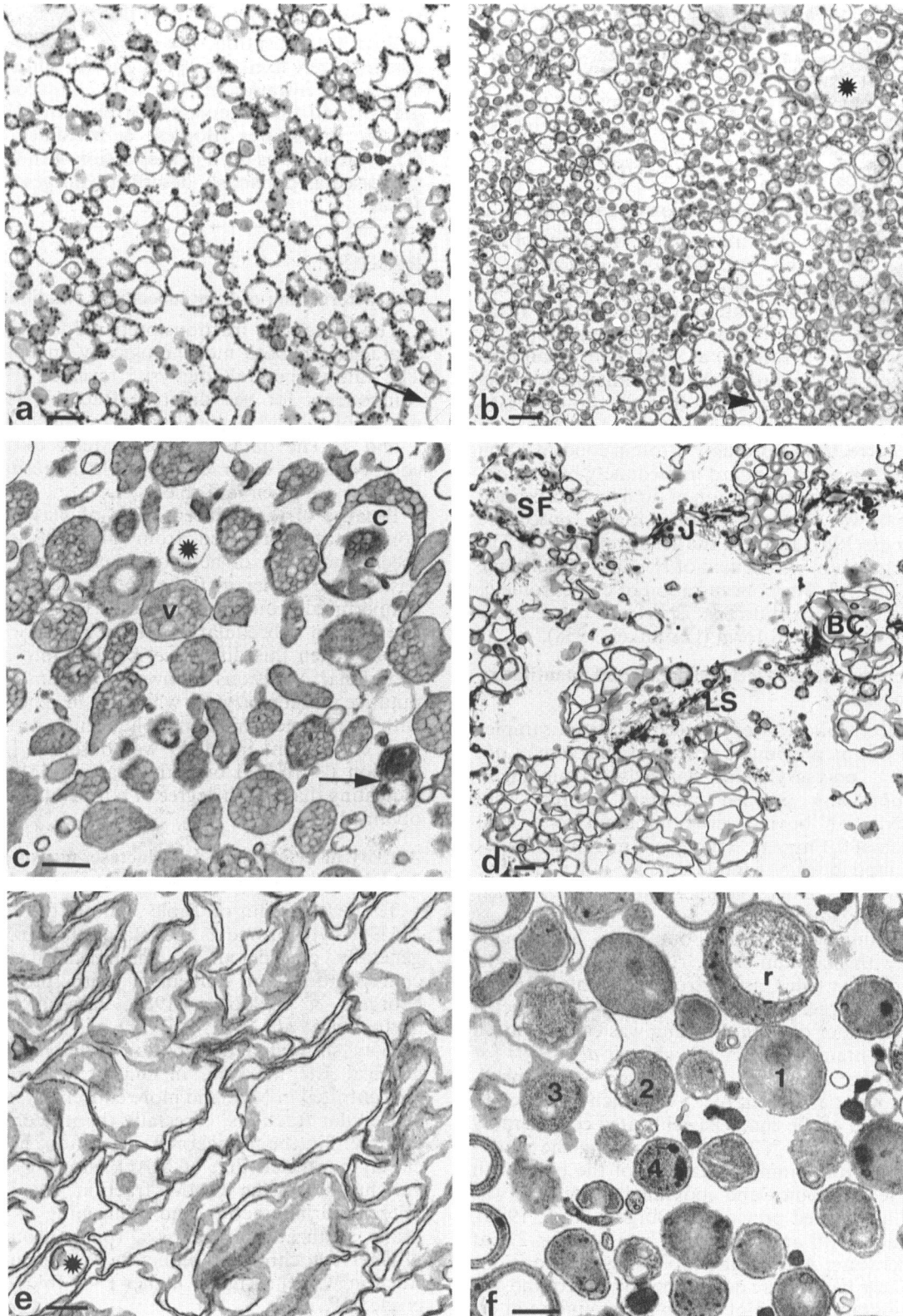


Fig. 1. Electron micrographs of liver subcellular fractions

The micrographs show fields close to the middle of sectioned pellets of the fractions. Bars in all panels are $0.25\ \mu\text{m}$. a, Rough microsomes. The fraction consists almost exclusively of ribosome-covered vesicles with a clear content. A few smooth-surfaced profiles are visible (arrow). b, Smooth microsomes. The fraction consists mainly of smooth-surfaced vesicles, of heterogeneous dimensions. The larger elements (asterisk) may derive from the plasma membrane. Golgi cisternae (arrowhead) are also contaminants of this preparation. c, G_{1+2} . This Golgi-enriched fraction contains mainly lipoprotein-filled vacuoles (v), some of which (c) appear to be connected to Golgi cisternae. Occasional empty profiles (asterisk) of undetermined origin and lysosomal contaminants (arrows) are also visible. d, Plasma membrane. The membrane sheets of this fraction contain portions

Table 1. Composition of rat liver subcellular fractions

Values are averages \pm S.E.M. (for averages of three experiments) or half-ranges (for averages of two experiments). Numbers in parentheses indicate number of experiments.

Fraction	Protein recovery (mg of protein/g of liver) [†]	Phospholipid (mg/mg of protein)	RNA (mg/mg of protein)
TH*	146 \pm 11 (7)	0.227 \pm 0.010 (7)	
MR	33.4 \pm 1.9 (5)	0.383 \pm 0.009 (3)	0.140 \pm 0.000 (2)
RMR	5.9 \pm 1.9 (3)	0.367 \pm 0.032 (3)	0.204 \pm 0.010 (2)
SMR	5.6 \pm 1.2 (3)	0.522 \pm 0.025 (3)	0.029 (1)
G ₁₊₂	0.330 \pm 0.06 (3)	0.937 \pm 0.014 (3)	
PM	1.17 (1)	0.430 (1)	
LYS	0.12 \pm 0.03 (2)	0.330 \pm 0.030 (2)	
MITO	8.4 \pm 0.8 (3)	0.187 \pm 0.003 (2)	
OMM	0.028 \pm 0.040 (2)	0.970 \pm 0.010 (2)	
IMM + matrix	2 (1)	0.160 (1)	

* Prepared from the perfused liver.

[†] Wet weight of liver before perfusion.

from 10% gels. Moreover, by increasing the electroblotting time from 90 to 180 min, the amount of reductase (microsomal or purified ws-red), detected on the nitrocellulose filter by radioimmunostaining increased by less than 10%. Reductase was efficiently retained on the filters, since a second sheet of nitrocellulose, placed behind the first one during electroblotting, contained less than 10% of the reductase found on the first sheet. Another point tested was the uniformity of the electric field in our blotting apparatus; we found that, regardless of the position of the sample on the gel, the efficiency of transfer of cytochrome *b₅* reductase to the nitrocellulose filter was the same (results not shown).

Fig. 2 shows the appearance of a blot obtained with rat liver total homogenate (TH) and subcellular fractions. In all fractions, only one band, running slightly behind ws-red, was stained. No staining was obtained when preimmune Ig was used (lane 2). By cutting out and counting areas containing the reductase band from blots like the one of Fig. 2, it was possible to estimate the reductase concentration of different subcellular fractions (see the Materials and methods section). The typical standard curve of Fig. 3(a) shows that the sensitivity of the assay was in the ng range. Fig. 3(b) shows how the amount of reductase measured in TH increased linearly with the amount of protein loaded onto the gel. Linearity of the assay was tested routinely on all samples.

Comparison of enzyme activity and radioimmunoblotting data

The reductase concentration, estimated by quantitative radioimmunoblotting in homogenates prepared from

perfused livers of male rats, was 1.66 ± 0.2 μ g/mg of protein (average of five experiments), in good agreement with the value reported by Takesue & Omura (1970), based on the recovery of enzyme activity in a purified reductase preparation (~ 1 μ g of reductase/mg of microsomal protein).

To compare the subcellular distribution of the antigen and the enzyme activity, we initially carried out differential centrifugation experiments, in which we prepared three crude fractions (a combined nuclear + MITO fraction, an MR fraction, and a high-speed supernatant) for our determinations. Fig. 4 shows that the distribution of antigen and enzyme among the fractions was different. Less of the antigen than enzyme activity was recovered in the MR fraction (33% versus 45% of total recovered), and this decrease was compensated by the higher antigen recovery in the high speed supernatant and especially in the combined nuclear + MITO pellet (62% versus 53%).

The results on purified fractions, presented in Table 3, confirm the difference in the subcellular distributions of antigen and enzyme activity. In this Table, the results of radioimmunoblotting determinations on the same fractions as those of Table 2 are presented and compared with the NADH-cytochrome *c* reductase activity data. It can be seen that the concentration of antigen in the MR fraction relative to that in the TH was lower than the enzyme r.s.a. in MR (20–25% lower), while it was higher in the MITO, LYS and PM fractions (columns 1 and 2). Thus, the ratios of antigen concentration in the latter fractions to that in MR were 1.5–2.0 times higher than the ratio for the enzyme activity. The higher ratio of

of the three domains of the hepatocyte plasma membrane, i.e. the lateral surface (LS), the bile canalicular surface (BC), and the sinusoidal front (SF). Junctional regions (J) between adjacent lateral membranes are visible. Abundant filamentous material, as well as adherent vesicles are associated with the membranes. e, Mitochondrial outer membranes. This fraction contains almost exclusively empty membrane sacs with complex shapes. The tightly packed elements have assumed complementary interdigitating forms. These interdigitations, when cut transversally, give rise to onion-like images (asterisk). f, Lysosomes. The predominant components of this fraction are heterogeneously shaped granules with an electron-dense content. The content may appear relatively homogeneous (1), more granular (2 and 3), or it may contain coarse deposits (4). Ring-shaped profiles (r) could derive from transverse sections of cup-shaped elements, or correspond to lysosomes containing engulfed portions of cytoplasm. Occasional empty smooth-surfaced vesicles and membrane fragments of undetermined origin can be seen.

Table 2. Relative specific activities of marker enzymes in rat liver subcellular fractions

Relative specific activities (r.s.a.) are activities/mg of protein in fractions divided by activity/mg of protein in the TH, \pm s.e.m. (for averages of three experiments) or half-ranges (for averages of two experiments). Numbers in parentheses indicate number of experiments; - indicates that the enzyme activity was not determined; UR (under-recovered) indicates that the assay was carried out but that the activity was not fully recovered. Specific enzyme activities (in nmol of product formed/min per mg of protein, except for cytochrome oxidase) in the TH prepared from the perfused liver were: NADPH-cytochrome *c* reductase, 27 ± 6 (six experiments); esterase, 1219 ± 173 (four experiments); galactosyltransferase, 0.49 ± 0.23 (two experiments); cytochrome oxidase, $3.8 \Delta 4$ units/min per mg of protein per ml (one experiment); MAO, 0.85 ± 0.12 (seven experiments); alkaline phosphodiesterase I, 11 (one experiment). The specific activities of the same enzymes in TH obtained from nonperfused livers were $\sim 25\%$ lower, because of the presence of blood proteins. Specific activities of arylsulphatase (three experiments) and β -galactosidase (two experiments) determined in the nonperfused TH were 25 ± 3.2 and 1.84 ± 0.08 nmol/min per mg of protein respectively.

Fractionation procedure	Fraction	NADPH-cytochrome <i>c</i> reductase (MR)*	Esterase (MR)	Galactosyl transferase (Golgi)	Cytochrome oxidase (IMM)	Monamine oxidase (OMM)	Alkaline phosphodiesterase I (PM)	Aryl sulphatase (LYS)	β -Galactosidase (LYS)
Mitochondria and mitochondrial sub-fractions	MR	3.2 ± 0.4 (2)	3.0 (1)	-	0.2 (1)	0.6 ± 0.0 (3)	-	-	-
	MITO	0.2 ± 0.1 (2)	0.1 (1)	-	2.3 (1)	2.1 ± 0.1 (3)	-	-	-
	OMM	0.8 (1)	0.3 (1)	-	0.06 (1)	34.7 ± 1.7 (2)	-	-	-
	IMM + matrix	-	-	-	3.1 (1)	UR	-	-	-
Golgi, rough and smooth microsomes	MR	3.7 ± 0.2 (3)	3.2 ± 0.3 (2)	1.7 ± 0.5 (2)	-	0.62 (1)	-	-	-
	RMR	3.4 ± 0.1 (3)	3.9 ± 0.3 (2)	0.5 ± 0.2 (2)	-	0.09 (1)	-	-	-
	SMR	5.3 ± 0.2 (3)	3.1 ± 0.8 (2)	7.9 ± 4.6 (2)	-	1.65 (1)	-	-	-
	G ₁₊₂	1.0 ± 0.2 (3)	0.4 ± 0.1 (2)	39.2 ± 7.6 (2)	-	1.25 (1)	-	-	-
Lysosomes	MR	3.0 ± 0.3 (2)	-	-	0.25 ± 0.10 (2)	0.78 ± 0.12 (2)	-	0.86 (1)	0.61 ± 0.15 (2)
	LYS	0.37 ± 0.03 (2)	-	-	0.65 ± 0.08 (2)	0.73 ± 0.04 (2)	-	63.7 (1)	65.6 ± 3.1 (2)
Plasma membrane	PM	1.3 (1)	1.2 (1)	-	-	0.46 (1)	23 (1)	-	-

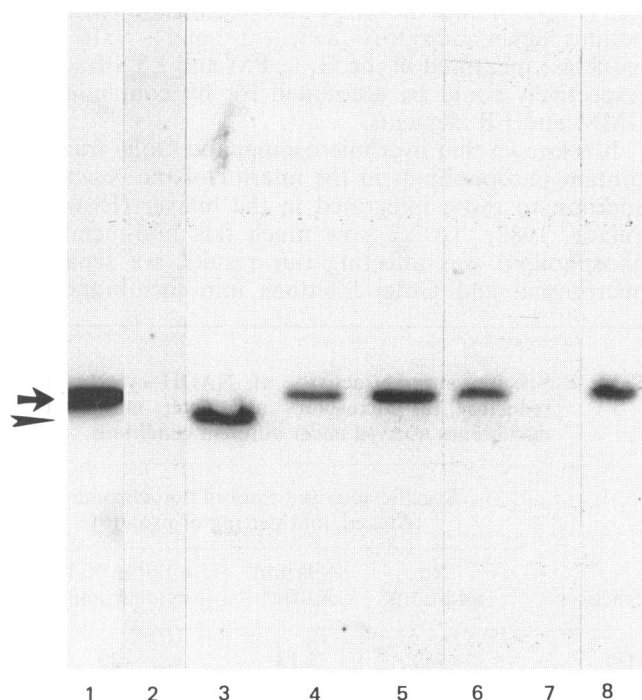
* The fraction in which the enzyme is most concentrated is indicated in parenthesis.

Table 3. Comparison between cytochrome *b₅* reductase enzyme r.s.a. and relative antigen concentrations in rat liver subcellular fractions

R.s.a. is specific enzyme activity (NADH–cytochrome *c* reductase) in fractions divided by specific enzyme activity in the TH, which was 330 ± 40 nmol/min per mg of protein (average \pm S.E.M. of five experiments), when homogenates were prepared from the perfused liver. Relative concentration is concentration of cytochrome *b₅* reductase antigen in fractions divided by its concentration in the TH, which was 1.66 ± 0.2 μ g/mg of protein (average \pm S.E.M. of five experiments), when homogenates were prepared from perfused livers. Values given are averages \pm half-range.

Fractionation procedure	Fraction	R.s.a. or relative concentration			
		Measured		Corrected*	
		Enzyme	Antigen	Enzyme	Antigen
Mitochondria and outer mitochondrial membrane (two experiments)	MR	2.06 ± 0.16	1.54 ± 0.22	1.83	1.23
	MITO	0.84 ± 0.03	0.88 ± 0.02	0.81	0.87
	OMM	13.7 ± 2.3	16.42 ± 1.18	15.33	18.46
Golgi, rough and smooth microsomes (two experiments)	MR	2.28 ± 0.08	1.79 ± 0.06	2.04	1.49
	RMR	1.75 ± 0.50	1.59 ± 0.30	1.71	1.55
	SMR	3.20 ± 0.17	2.34 ± 0.19	2.56	1.56
	G ₁₊₂	1.68 ± 0.21	1.66 ± 0.23	1.06	1.01
Lysosomes (two experiments)	MR	2.5 ± 0.3	1.76 ± 0.03	2.20	1.39
	LYS	0.39 ± 0.10	0.56 ± 0.11	0	0.06
Plasma membrane (one experiment)	PM	0.70	0.97	0	0.37

* These columns give the values for NADH–cytochrome *c* reductase r.s.a. and reductase antigen relative concentration after correction for the contribution of contaminating MR and OMM to the fractions. The correction procedure is explained in the Appendix.

**Fig. 2. Radioimmunostaining with antireductase antibodies of a Western blot of different liver subcellular fractions**

Liver subcellular fractions and ws-red were electrophoresed on a 10% polyacrylamide gel, the gel was then electrophoretically blotted onto nitrocellulose, and the resulting blot was processed as described in the Materials and methods section. Lanes contained the following samples: 1 and 2, TH (5 μ g of protein); lane 2 was

mitochondrial to MR antigen concentration was, of course, reflected in the results obtained with purified OMM, where there was nearly 11-fold more antigen per mg of protein than in MR, as compared with only 7-fold more enzyme specific activity.

To test whether the enzyme assay was not truly reflecting the reductase concentrations in subcellular fractions because of differences in intermediate acceptor (cytochrome *b₅*) concentration or availability, the NADH–cytochrome *c* reductase activity was assayed in the OMM and MR fractions in the presence of added cytochrome *b₅* (Table 4). In this experiment, we first abolished the activity of endogenous intermediate acceptor by solubilization with Triton X-100 (compare columns 2 and 1 of Table 4). The NADH–cytochrome *c* reductase activity was then restored by the addition of high concentrations of exogenous cytochrome *b₅*. When equal concentrations of the water-soluble fragment of cytochrome *b₅* were added to the detergent-solubilized OMM and MR samples, the activity was more efficiently restored in the OMM than in the MR fraction (column 3, Table 4), so that the ratio of the activity in OMM to that in MR attained a value of nearly 12, in good agreement with the radioimmunoblotting data.

incubated with preimmune Ig (3 μ g/ml) instead of anti-reductase antibodies; 3, purified ws-red (10 ng); 4 and 5, MR (1.5 and 3 μ g of protein, respectively); 6, MITO (2 μ g of protein); 7, IMM + matrix (2.2 μ g of protein); 8, OMM (0.2 μ g of protein). The arrow and arrowhead indicate the positions of the reductase and its water-soluble fragment, respectively. Exposure and photography of all lanes were the same.

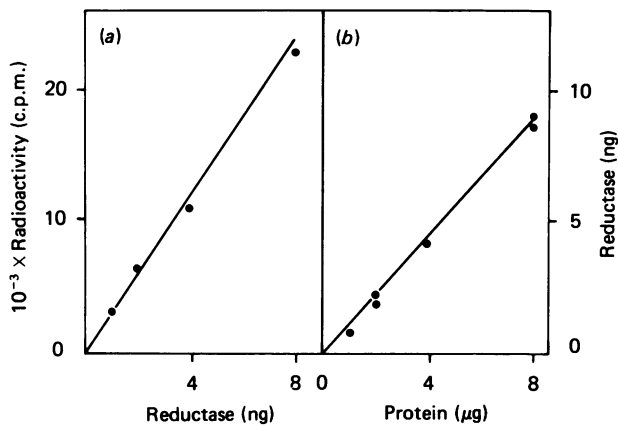


Fig. 3. Quantitative radioimmunoassay for cytochrome *b*₅ reductase

(a) Shows a typical standard curve, obtained with four quantities of purified ws-red. Each point represents a single determination. (b) Shows the results of an experiment in which increasing amounts of liver TH (abscissa) were assayed for reductase content (ordinate) with the radioimmunoassay.

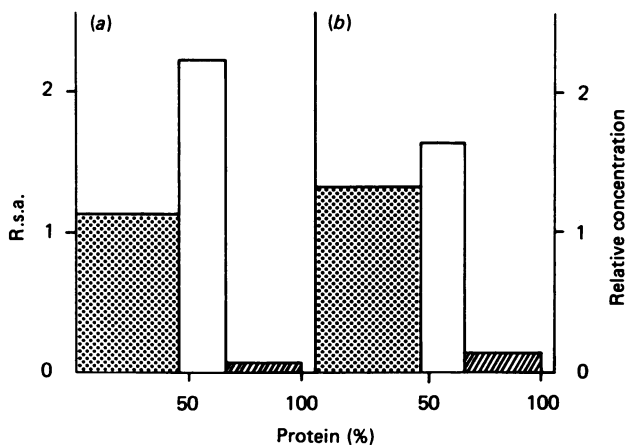


Fig. 4. Distribution of NADH-cytochrome *c* reductase activity and of reductase antigen between liver subcellular fractions prepared by differential centrifugation

A non-perfused liver homogenate in 0.25 M-sucrose was fractionated into a crude nuclear+mitochondrial pellet (stippled box; 25000 *g*_{max.} for 10 min), a MR pellet (open box; 200000 *g*_{max.} for 1 h), and a final supernatant (hatched box). The resulting fractions were assayed for protein, NADH-cytochrome *c* reductase activity (a), and cytochrome *b*₅ reductase antigen (b). The width of the boxes is proportional to the percentage of recovered protein found in each fraction, while the height is proportional to the r.s.a. (% of recovered NADH-cytochrome *c* reductase enzyme activity/% of recovered protein) (a) or relative concentration of cytochrome *b*₅ reductase (% of recovered antigen/% of recovered protein) (b). The area of the boxes is proportional to the fraction of reductase activity or antigen recovered in the fractions. Total recovery of protein, NADH-cytochrome *c* reductase activity, and cytochrome *b*₅ reductase antigen were 83.6, 85.5, and 88.3% of that in the TH, respectively.

In Table 3, we have also presented relative antigen concentrations and enzyme activities for each subcellular fraction after correction for cross-contamination between cell fractions, using the marker enzyme data of Table 2 (see the Appendix for an explanation of the correction procedure). After correction, the ratio of antigen concentration in OMM to that in MR reached a value of 15. While all of the reductase enzyme activity in the LYS and PM fractions could be accounted for by cross-contamination, the radioimmunoassay data indicated that some of the antigen present in these fractions could be endogenous to LYS and PM respectively. Finally, after correction for cross-contamination, the antigen concentrations in rough and smooth MR became nearly the same.

In Fig. 4, we have expressed the antigen concentration in our subcellular fractions on a phospholipid basis, to give an idea of the reductase concentrations per membrane surface area in different organelles. Because the phospholipid content of RMR and SMR is considerably lower than that of OMM (see Table 1), the difference in reductase concentration between the OMM and the submicrosomal fractions became considerably less when expressed on the basis of phospholipid rather than protein content. Nonetheless, the reductase concentration on a phospholipid basis in OMM was still ~5-fold higher than that in SMR and RMR (Fig. 5a). Even lower concentrations were found in the G₁₊₂, PM and LYS fractions, while no reductase could be detected in the IMM+matrix fractions (see Fig. 2). The differences between the fractions became even more marked after correction of the data for cross-contamination (Fig. 5b), since the reductase concentration in OMM became higher and since ~1/3, ~2/3 and ~9/10 of the reductase measured in the G₁₊₂, PM and LYS fractions respectively could be accounted for by contaminating OMM and ER elements.

It is known that liver microsomal and Golgi fractions contain phospholipids in the interior of the vesicles, in addition to those integrated in the bilayer (Howell & Palade, 1982). To see how much this non-membrane phospholipid was affecting our results, we separated microsomal and Golgi fractions into membrane and

Table 4. Specific enzyme activity of NADH-cytochrome *c* reductase in microsomes and outer mitochondrial membranes assayed under different conditions

Fraction	Specific activity (nmol of cytochrome <i>c</i> reduced/min per mg of protein)		
	No additions	+ Triton X-100*	+ Triton X-100 + cytochrome <i>b</i> ₅ †
MR	469	3.13	129
OMM	3821	N.D.	1533
OMM/MR	8.1		11.9

* Samples were preincubated in 0.8 ml of NADH-cytochrome *c* reductase assay mixture containing 1% Triton X-100 for 5 min at 0 °C before beginning the reaction by addition of NADH.

† Samples were preincubated in 0.8 ml of NADH-cytochrome *c* reductase assay mixture containing 1% Triton X-100 and 3.2 μM-cytochrome *b*₅ for 5 min at 0 °C before beginning the reaction by addition of NADH.

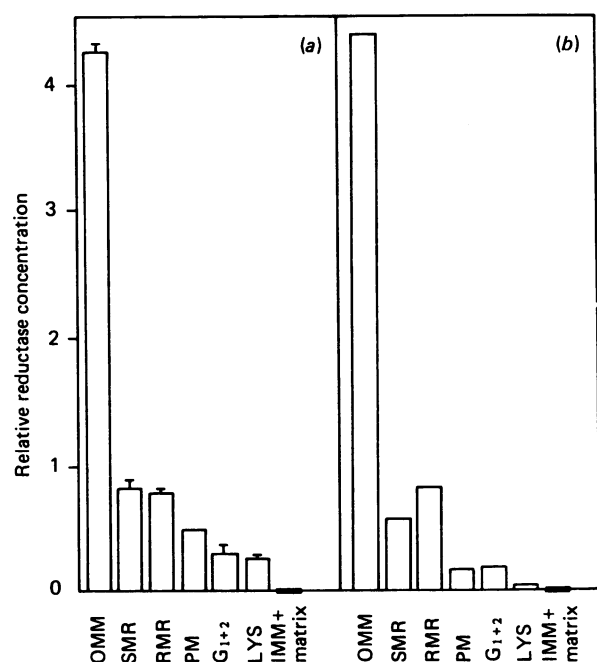


Fig. 5. Heterogeneous distribution of cytochrome *b*₅ reductase antigen among liver subcellular membranes

The relative reductase antigen concentrations in liver subcellular fractions are expressed in this Figure on a phospholipid basis: the relative reductase concentration (ordinate) is antigen concentration ($\mu\text{g}/\text{mg}$ of phospholipid) in the fraction divided by antigen concentration ($\mu\text{g}/\text{mg}$ of phospholipid) in the TH. Values shown are averages of two experiments (except for PM: one experiment). Bars represent half-ranges. (a) Shows the uncorrected values; (b) shows the values obtained after correction for cross-contamination of the fractions, as explained in the Appendix.

Table 5. Distribution of microsomal and Golgi components between pellets and supernatants after pH extraction

Samples were treated with 0.1 M- Na_2CO_3 and separated into a high speed pellet and supernatant as described by Fujiki *et al.* (1982). Values are given as a percentage of the sum of the amounts recovered in pellet + supernatant.

Fraction	Recovered in pellet (% of total)		
	Protein	Phospholipid	Reductase*
RMR	45.8	97.9	95.0
SMR	64.7	95.9	94.6
G ₁₊₂	54.7	89.7	ND†

* Assayed by quantitative radioimmunoblotting.

† Not determined.

content subfractions by alkaline (pH 11) extraction followed by differential centrifugation, and determined the distribution of protein, phospholipid, and reductase in these subfractions. As can be seen from Table 5, negligible amounts of phospholipid were released from the microsomal fractions by alkaline extraction, while about 10% of the G₁₊₂ phospholipid was extracted.

Thus, the reductase concentration per membrane phospholipid in G₁₊₂ is 10% higher than shown in Fig. 5. Table 5 also shows that reductase was resistant to alkaline extraction, confirming its nature as a tightly bound, integral membrane protein.

DISCUSSION

In this study, we have reinvestigated the intracellular distribution of rat liver cytochrome *b*₅ reductase, determining its levels in rat liver subcellular fractions with the classical enzyme assay as well as with a quantitative radioimmunoblotting method. Advantages of the radioimmunoblotting method over other immunological assays are its suitability for the study of membrane proteins, the lack of interference of cross-reactive or contaminating antigens, and the low consumption of antibody with excellent sensitivity. Using this assay, we found that the intracellular distribution of reductase antigen was different from that of the enzyme activity, indicating that caution should be exercised in equating enzyme activities to enzyme concentrations in cell fractionation studies. Comparison between immunological and spectroscopic or enzymic assays have been carried out by others (Negishi & Kreibich, 1978; Shawver *et al.*, 1984) on rat liver microsomes, and a discrepancy between the microsomal concentration of cytochrome *b*₅ measured spectroscopically and immunologically has been reported (Shawver *et al.*, 1984). To our knowledge, however, this is the first time that such a comparison has been carried out in a comprehensive cell fractionation study.

The most striking result of this study is the high concentration of reductase in OMM relative to all other membranes, including those of the ER. There was 11-fold more reductase/mg of protein and 5-fold more reductase/mg of phospholipid in the OMM than in the MR fraction. Although higher concentrations of reductase in OMM than in MR fractions had previously been reported (Ito *et al.*, 1981; Sottocasa *et al.*, 1967), differences as large as the one observed here had not been seen. The discrepancy between our results and those of other authors can be attributed to the following three factors. (1) The high ratio (0.4) of NADH-cytochrome *c* enzyme activity in the starting MITO to the activity in MR. This ratio is higher than that reported by several groups (e.g., Amar-Costesec *et al.*, 1974), but in agreement with that found by others (Ito *et al.*, 1981; Sottocasa *et al.*, 1967). (2) The high degree of purity of our OMM preparation resulted in a large enrichment of NADH-cytochrome *c* reductase activity (18-fold) over the starting MITO. (3) When measured as antigen, an even higher concentration of reductase in OMM relative to that in ER membranes was revealed. The results of experiments in which exogenous cytochrome *b*₅ was added to the enzyme assay mixture, suggest that the discrepancy between NADH-cytochrome *c* reductase activity and antigen concentration could be entirely accounted for by differences in intermediate acceptor concentration or availability in the two compartments.

It should not be concluded on the basis of our data that most of the reductase in liver is present on OMM. In fact, because of the much larger size of the ER than the OMM compartment, a larger proportion of reductase resides in ER membranes, notwithstanding the lower concentration of the enzyme in that location. Thus, on

the assumption that the subcellular fractions were representative of the organelle populations from which they were derived, it can be calculated (Tables 1 and 2) that roughly 50% of reductase antigen is present in the ER, 40% in the OMM compartment, and 10% elsewhere.

Three fractions, G_{1+2} , PM and LYS, had very low concentrations of reductase, although in PM and LYS the amounts measured as antigen were substantially higher than those detected with the enzyme assay. An obvious question is whether the reductase measured in these fractions is endogenous to the membranes of these organelles, or whether it was contributed by contaminating membranes. Our calculations suggest that, although substantial amounts are due to contamination, low levels of reductase are indeed endogenous to the organelles in question. Moreover, in a previous study (Borgese & Meldolesi, 1980), we demonstrated that at least a part of the reductase activity in the G_{1+2} fraction is present on a subpopulation of membranes, distinguishable from contaminating ER and OMM on the basis of the perturbability of their buoyant density by digitonin. Nonetheless, we cannot decide on which membranes the reductase is residing within the G_{1+2} fraction, since liver Golgi fractions are known to be quite heterogeneous, containing also lipoprotein-filled endosomes (Kay *et al.*, 1984). Regarding the possibility of the presence of the reductase on the PM, Wibo *et al.* (1981) showed that in liver PM fractions none of the NADH-cytochrome *c* reductase activity was associated with digitonin-perturbable membranes, and concluded that its presence was due to cross-contamination. A similar conclusion would be derived from the cytochrome *c* reductase activity data reported in the present study. However, on the basis of our immunological data, we feel that it is likely that some reductase is endogenous to liver PM. It should be recalled that the reductase can localize to plasma membranes of other cells, as demonstrated by the presence of the antigen on erythrocyte ghosts (Borgese *et al.*, 1982). We do not, however, wish to stress this point any further here, because the important conclusion of this study is that there are very large differences in the concentration of reductase in different compartments (at least 16-fold between the two extremes, OMM and LYS), regardless of whether lysosomes or plasma membranes contain low levels of the enzyme. The question then, is how the cell regulates the concentration of this postrationally inserted protein in different organelles.

The different concentrations of reductase in different membranes could be caused by different rates of insertion and/or different rates of degradation of the enzyme. The data presented in this paper, combined with the results of our previous studies on the biosynthesis of the reductase, indicate that both these mechanisms may be operating. We previously demonstrated that cytochrome *b₅* reductase is inserted post-translationally and directly into a variety of membranes (Borgese & Gaetani, 1980, 1983; Borgese *et al.*, 1980) and that the reductase within the G_{1+2} fraction is degraded with roughly the same rate constant as the enzyme in the ER. Therefore, on the basis of the finding reported here, that there is a 3-fold difference in reductase concentration between membranes in the MR and G_{1+2} fractions, we must conclude that the enzyme inserts roughly 3-fold less

rapidly (per phospholipid bilayer area) into membranes of the G_{1+2} fraction than into ER membranes. In the same study quoted above (Borgese *et al.*, 1980), we found that the reductase in OMM is degraded with a rate constant half that of the enzyme in ER. This slower degradation rate would cause a 2-fold higher reductase concentration in membranes of the OMM than of the ER, if the rates of insertion of the enzyme per bilayer surface area of the two compartments were the same. Since the data presented here indicate a 5-fold higher reductase concentration in OMM over ER membranes, the reductase must insert two to three times more rapidly (per phospholipid bilayer area) into the former than into the latter membranes.

Although we can conclude that the post-translational insertion of the reductase is a non-random event, we do not at present know where the basis for this selectivity lies. On the basis of the results of biochemical and immunological experiments (Meldolesi *et al.*, 1980; Kuwahara *et al.*, 1978), it is thought that the microsomal and mitochondrial forms of the reductase are identical; however it cannot be excluded that minor difference exist between the two reductases which would cause them to be targeted to different membranes.

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APPENDIX

Procedure for obtaining the corrected values of NADH–cytochrome *c* reductase r.s.a. and reductase antigen relative concentration given in Table 3

Corrections are based on the marker enzyme data of Table 2, and carried out as follows. First, corrected values for MAO and reductase in OMM have been calculated. Assuming that the r.s.a. of cytochrome oxidase and esterase in the IMM+matrix and MR fractions respectively approximate the values which would be found in the 100% pure fractions, the contributions of protein of IMM+matrix and of ER elements to the OMM fraction are calculated to be 2% and 10% of the total protein respectively. Assuming that the presence of other contaminants in the OMM fraction is negligible, the remaining protein (88%) would be contributed by OMM. Thus, the r.s.a. of MAO in a pure OMM fraction would be $34.7/0.88 = 39.4$. To calculate the corrected values for reductase r.s.a. and relative concentration in OMM, the uncorrected values of 2.06 and 1.54 for the r.s.a. and relative concentration respectively of the enzyme in MR have been used. For example, corrected r.s.a. of the reductase in OMM is:

$$\frac{13.7 - (2.06 \times 0.1)}{0.88} = 15.3$$

where 13.76 and 2.06 = observed r.s.a. of NADH–cytochrome *c* reductase in OMM and MR fractions

respectively, 0.1 = fraction of protein in OMM contributed by ER elements, 0.88 = fraction of protein in OMM contributed by *bona fide* OMM. The ratio of corrected reductase r.s.a. or relative concentration to MAO corrected r.s.a. in OMM has been used to subtract the reductase activity or concentration contributed by OMM to all other fractions, except MITO. In these calculations, the protein contribution of OMM to the other fractions has been neglected. Values in G_{1+2} , MITO, LYS and PM have also been corrected for microsomal contamination. In this case, the protein contribution of MR to these fractions has been considered. For G_{1+2} , MITO and PM, esterase has been considered as the ER marker, and the ratio of the corrected reductase values to esterase in MR in each group of experiments has been used to calculate the contribution of MR reductase to the activities or concentrations found in the above fractions. Since in the PM experiment the MR fraction was not prepared, the average values for esterase and corrected reductase r.s.a. or concentration of MR from all other experiments with perfused livers have been used for the calculations. In the LYS experiments, esterase assays were not carried out, and NADPH–cytochrome *c* reductase has been used as the MR marker.